AD_____

Award Number: DAMD17-03-1-0224

TITLE: Angiogenesis and Therapeutic Approaches to NF1 Tumors

PRINCIPAL INVESTIGATOR: David F. Muir, Ph.D.

CONTRACTING ORGANIZATION: University of Florida
Gainesville, Florida 32611-5500

REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Angiogenesis and Therapeutics Approaches to NF1 Tumors

5. FUNDING NUMBERS
DAMD17-03-1-0224

6. AUTHOR(S)
David F. Muir, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Florida
Gainesville, Florida 32611-5500

E-Mail: muir@ufbi.ufl.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The main goal of this project is to specify how anti-angiogenic approaches can be effectively applied to NF1 tumors. To this end, we will first determine whether NF1 heterozygosity alters the responsiveness of endothelial cells to angiogenic regulators. We will test if Nf1-/+ endothelial cells are particularly responsive to pro-angiogenic factors produced by NF1 tumor cells and, perhaps even more importantly, which anti-angiogenic factors are most effective in abrogating the angiogenic response evoked by NF1 tumors. In particular, endostatin will be thoroughly examined as a potential anti-angiogenic therapy for NF1 tumors. Gene therapy for NF1 tumors has not been tested due to the lack of an appropriate NF1 tumor model. We have established a working xenograft model of neurofibroma in the mouse in which the efficacy of endostatin gene therapy will be accessed. This model involves the initiation of neurofibromas by implantation of human NF1 tumor-derived, neurofibromin-null Schwann cells into the nerves of mice with an Nf1-/+ background. Tumor progression and vascularity will be assessed in vivo by MRI non-invasive imaging. MRI data will be corroborated by end-point histology using precise labeling of tumor and host cell components.

14. SUBJECT TERMS
Cancer biology, angiogenesis, xenograft, gene therapy, anti-angiogenesis therapy, MRI

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common genetic disease with a wide variety of features which primarily involve the nervous system and related tissues. NF1 is characterized by abnormal cell growth and a high incidence of neurofibroma, a nerve tumor composed predominantly of Schwann cells. Plexiform neurofibromas often grow very large and are debilitating or fatal to NF1 patients. Thus, there is a serious need for better therapies to manage NF1 tumor growth. To this end, we have developed and exploited two animal models of NF1. The first involves a strain of mice in which the *Nf1* gene was functionally deleted. These Nf1 knockout mice are a valuable model for examining the biology of Nf1 tissues both in vivo and in vitro. Secondly, we have cultured tumor cells from human NF1 tumors. These human cell lines form neurofibroma-like tumors when implanted into the mouse nerve. Using these resources and animal models we can examine the formation of NF1 tumors under controlled conditions. The Aims of this proposal are to determine how NF1 tumors induce the formation of new blood vessels and to test therapies to inhibit this process as an means to stop tumor growth.

There is considerable heterogeneity in the vasculature found in different tissue and tumor types. The first Aim of this work is to determine whether blood vessel formation might be altered in NF1 patients. For this we will use the Nf1 knockout mouse. Endothelial cells will be cultured from wild-type and Nf1-/+ mouse tissues. The ability of these cells to form blood vessels in response to pro-angiogenic and anti-angiogenic factors will be tested in tissue culture assays. Important differences in the responsiveness of Nf1 endothelial cells will be confirmed using in vivo assays conducted in wild-type and Nf1 knockout mice.

We have established and characterized numerous cell cultures from human NF1 tumors, many of which have been grown as tumor grafts in the nerves of Nf1 mice. We will test the hypothesis that the rate of growth by these NF1 tumor xenografts is associated with the degree of newly formed vasculature. Also, comparisons will be made between xenografts implanted in normal mice and Nf1 mice. In vivo tumor growth and vascularity will be correlated with the expression of angiogenic regulators by the implanted cell lines. These experiments will test the hypothesis that tumor growth and invasion is dependent on the responsiveness of Nf1 endothelial cells and other reactive cells in the nerve that contribute to tumor formation.

There are several anti-angiogenic factors that show excellent promise as potent inhibitors tumor growth. In this aim we will test endostatin as an anti-tumor treatment for peripheral nerve tumors in NF1. This Aim will be expanded to include other anti-angiogenic therapies based on discoveries made in the Aims described above. Gene therapy using endogenous angiogenic inhibitors, like endostatin, is considered by many to be the most promising approach to bring the anti-angiogenic therapy into the clinic. As a simplified experimental model, we will examine the growth and vascularity of tumor xenografts that are engineered to produce endostatin. Second, using a strategy more relevant to clinic treatment, we will apply an endostatin-viral vector (AAV-endostatin) to NF1 tumors already growing in the mouse. In both treatment models, growth and regression of tumor and neovasculature will be monitored in vivo by non-invasive magnetic resonance imaging (MRI) followed by autopsy examination of the tumor tissues. Our overall goal is to discover effective therapies for the treatment of plexiform neurofibromas by blocking the ability of these aggressive tumors to recruit the blood vessels required for their growth.

BODY

Technical Objective 1: EXAMINE THE RESPONSE OF NF1 ENDOTHELIAL CELLS TO ANGIOGENIC REGULATORS.

Task 1: Perform in vitro assays of Nf1+/- endothelial cell responses to pro-angiogenic factors:

Progress: In this aim we are testing the hypothesis that the in vitro response of Nf1+/- endothelial cells to pro-angiogenic factors differs from that of wild-type endothelial cells. To perform this aim it is first necessary to develop methods for the reliable culture of endothelial cells from Nf1+/- knockout mice. We increased the breeding of this mouse strain to meet our needs, and methods for genotyping the offspring were also improved.

We have tested several methods and modifications for the culture of brain microvessel endothelial cells from Nf1+/- and Nf1+/+ (wild-type) mice. Initially neonatal mice were used as a source of brain microvessels based on reports that neonatal endothelial cells proliferated readily and cultures were more easily expanded. Although our cultures expanded in response to endothelial cell mitogens, the contamination by other cell types was unacceptable. Further analyses indicated that neonatal capillaries did not adequately withstand the mechanical isolation procedures required to collect them intact separate from other dissociated cells. Subsequent testing showed capillaries from 4-5 week old mice were more easily isolated intact. Thus far we have mainly focused on establishing endothelial cell cultures from 4-5 week wild-type mice. Cell purity was assessed by immunostaining for the endothelial markers CD31 and von Willebrand's factor. We have achieved enriched cultures containing over 80% endothelial cells, and are improving the purity in each preparation. One preparation from Nf1+/- mice was performed with similar results. We are testing several mitogen treatments and culture substrata to enhance the proliferation and expansion of the endothelial cells to achieve increased numbers and higher purity of endothelial cells. These include b-FGF, VEGF, laminin, fibronectin, collagen IV and Matrigel. We are confident that our culture methods will soon yield endothelial cells of sufficient purity (>95%) and in sufficient numbers to begin assays for responses to pro-angiogenic factors.

In the interim, we obtained a immortalized mouse brain endothelial cell line (commercially available). Although this cell line is not useful as a true wild-type control, it has enabled us to develop methods and parameters for the in vitro pro-angiogenic assays.

In summary, we have made very good progress in establishing highly purified endothelial cultures from wild-type and Nf1+/- mouse brain microvessels. However, assay quality cultures are only now being produced and readied for testing responses to pro-angiogenic factors.

Task 2: Perform in vitro assays of Nf1+/- endothelial cell responses to anti-angiogenic factors:

Progress: As described for Task 1, prerequisite endothelial cultures and assay methods are nearly ready to begin testing responses to pro- and anti-angiogenic factors.

Task 3: Perform in vivo assays for angiogenesis in Nf1+/- knockout mice:

Progress: It this aim we are testing the hypothesis that the angiogenic responses of mice with an Nf1 background differ from wild-type mice. The main goal of our in vivo angiogenesis assays has been accomplished. Developing, executing and analyzing in vivo assays for angiogenesis has been challenging and labor intensive. First, we established an in vivo angiogenesis assay that involves exposing newborn mice to an elevated oxygen atmosphere for 1 week followed by a return to normal atmosphere. The latter

evokes a hypoxic response, including retinal neovascularization. New vessel formation was assessed by histological examination of sectioned retinas. Sixty-three mice (126 retinas) from 12 litters were examined. Results showed that neovascularization in retinas from Nf1+/- mice was 51% greater than in wild-type litter mates ($p \leq 0.008$).

To corroborate these encouraging findings, a second in vivo assay was developed involving the implantation of pro-angiogenic factors to the mouse cornea (which is naturally avascular). The angiogenic factor FGF-2 was bound to microbeads and implanted into a minute slit in the cornea. After 5 days, new vessel formations were scored by CD31 immunolabeling of whole mount corneas. Results showed the neovascular response of Nf1+/- mice was 67% greater than that of wild-type controls ($n=30$, $p \leq 0.000014$). Taken together these results provide convincing in vivo evidence that Nf1+/- mice have a significantly heightened angiogenic response to both hypoxia and the pro-angiogenic factor FGF-2. This finding indicates that heightened angiogenesis may play an important role in tumor development in NF1 patients and provides a foundation and justification for exploring anti-angiogenic therapies for neurofibroma.

Task 4: Determine the angiotrophic potential of human tumor cell lines:

Progress: This task involves collecting extracts from NF1 tumor cultures and normal Schwann cell control cultures for testing in angiogenic assays. As stated for Task 1, our in vitro assays using endothelial cell cultures are nearly established, but have not yet been ready for quantitative tests. On the other hand, we have collected and stored extracts from numerous NF1 tumor cultures and controls. In summary, although we have not yet determined the angiotrophic potential of human NF1 cell lines, sample extracts are ready and, as the endothelial cultures become available, we expect rapid completion of this work.

Technical Objective 2: EXAMINE THE VASCULARITY AND ANGIOGENIC PROPERTIES OF NF1 TUMOR XENOGRAFTS.

Task 1: Develop MRI imaging of tumor growth and vascularity:

Progress: Our goal in this aim is to establish methods and parameters for MRI imaging of tumor growth and recession in a xenograft model of neurofibroma. Schwann cell cultures from NF1 patient tumors are implanted in the nerves of mice with an Nf1 background. We have established and imaged numerous xenografts using various MRI parameters including T1 and T2-weighting. Also, the vascular properties of tumor xenografts were imaged using gadolinium enhancement. These image sets have been compiled and processed as 3D composites. Images and composites have been quantified using dynamic contrast enhancement plots, in which tumor values exceed normal surrounding nerve and muscle by over 40%, which is more than sufficient for excellent discrimination of tumor masses and margins. Taken together, we have made excellent progress in imaging tumor xenografts by MRI and are ready to apply these techniques to monitor tumor growth and, in particular, tumor regression in response to anti-angiogenic treatments as required for subsequent aims.

Task 2: Develop volumetric MRI and histology methods for tumor quantitation:

Progress: We have made excellent progress in the development of volumetric methods for quantitation of tumor growth by MRI and histology. As stated above, MRI imaging is now established and the data

sets have been and continued to be studied by post-hoc image analysis. We made 3D renderings of many tumor image sets (from consecutive slices) and have performed volumetrics to indicate tumor size. Methods and software refinements were tested and volumes obtained. We are still learning about and setting criteria for defining tumor margins that specify areas of established tumor, growing margins and even regression. This is an ongoing aspect of this work that evolves along with improved imaging and image interpretation. In summary, we have learned how to calculate tumor volume from 3D composites and established initial criteria for volume calculations that correlate well between MRI and histology assessments. Although applying this to subtle aspects of tumor growth is an evolving skill, our methods are in place to assess tumor volume for conservative the quantitative scoring required in subsequent aims.

Task 3: Quantify the growth and neovascularity of NF1 tumor xenografts:

Progress: The development of MRI and histology imaging methods involve a continuing effort to improve and refine image quality, discrimination and quantitation. Our goal is to analyze and compare the growth and vascularity of various xenografts with different growth properties. Thus far we have focused on grafting a subset of well characterized cell lines. Thus far, we developed two distinct xenograft models for NF1 tumors by engraftment of Schwann cell lines with varying tumorigenic properties, one representing plexiform neurofibroma and the other malignant peripheral nerve sheath tumor. Tumor growth has also been examined in animals by MRI at 2-week intervals, and tumor progression clearly delineated. In vivo quantitation is presently being compared with post-mortem histology. Vascularity assessed by in vivo gadolinium enhancement is being compared with vascular scoring of immunostained tissue sections. In summary, we have made excellent progress in the quantitation of tumor growth and vascularity by in vivo MRI imaging and are now performing histological correlations.

Because we have clearly established two distinct xenograft models, an immediate goal is to reliable discriminate these different tumor types using MRI, which represents an important aim for our experimental work and certainly has clinical importance as well. However, this effort has delayed our work involving the xenograft of additional NF1 tumor cell lines.

Task 4: Determine the effect of the Nf1+/- background on NF1 tumor xenografts:

Progress: This aim involves examining the growth of human cell line xenografts in wild-type and mice with an Nf1 background. Our aim is to determine if the host Nf1 background has an effect on tumor growth and if Nf1+/- cells might promote tumor growth compared to wild-type cells. Thus far, we examined the growth of two human NF1 tumor xenografts in Nf1+/- and wild-type mice. Tumors (n=6) were examined for extent of growth (size), proliferation, vascularity and infiltration of mast cells. The first two properties are responses of the engrafted cells and the latter two properties are responses of the host cells. There was a marked difference between the growth and proliferation rates in the two xenografts (sNF94.3 and sNF96.2). However, within each xenograft model no significant difference in growth (tumor size or proliferation rate) was observed in the host variants. Similarly, no difference were found in the extent and distribution of mast cells in and around the xenoplants. The vascular elements have not yet been scored. In summary, tumor size, proliferation and mast cell response do not appear different for the sNF94.3 and sNF96.2 xenografts. Additional xenografts need to be tested.

Task 5: Examine the angiogenic properties of Nf1+/- host cells within the NF1 tumor xenograft:

Progress: In this aim we will examine the possible tumorigenic and angiotrophic contributions of other Nf1-/+ host cell types. We have already examined the distribution of host mast cells associated with the xenografts and found, while these reactive cells accumulate around the tumors, they rarely infiltrate the tumor cell mass. Next, we plan to examine mast cell association with neovascular elements at the tumor margins. We have not yet performed any immunocytochemical labeling to determine the expression of angiotrophic factors by reactive mast cells. In summary, only preliminary work on this aim has been performed.

Technical Objective 3: EXAMINE THE EFFECTS OF ANGIOGENIC INHIBITORS ON THE GROWTH AND NEOVASCULARIZATION OF NF1 TUMOR XENOGRAFTS IN NF1+/- MICE.

Task 1: Transduce endostatin in NF1 tumor cultures:

Progress: No work as been done on this aim.

Task 2: Determine the effect of endostatin in vitro transduction on NF1 tumor xenografts:

Progress: No work as been done on this aim.

Task 3: Develop in vivo delivery of AAV-endostatin:

Progress: No work as been done on this aim.

Task 4: Assess the effect of endostatin delivery to established NF1 tumor xenografts:

Progress: No work as been done on this aim.

KEY RESEARCH ACCOMPLISHMENTS

1) Developed methods to culture brain microvessel endothelial cells from Nf1 and wild-type mice.
2) Determined that the in vivo neoangiogenic response to hypoxia is heightened in retinas of Nf1+/- mice compared to wild-type mice.
3) Determined that the in vivo neoangiogenic response to FGF-2 is heightened in the corneas of Nf1+/- mice compared to wild-type mice.
4) Established and documented two valid xenograft models of NF1 plexiform neurofibroma and malignant peripheral nerve sheath tumors.
5) Established methods and assessed tumor growth and vascularity of NF1 tumor xenografts.
6) Quantified tumor growth by two xenografted NF1 tumors.
7) Imaged and quantified vascularity of xenografted tumors using MRI, gadolinium permeability and dynamic contrast enhancement.

REPORTABLE OUTCOMES

Manuscripts: None

Abstracts:
G. Perrin, M. Wallace and D. Muir. 2003. Characterization of a reproducible xenograft model for NF1 plexiform neurofibroma. National Neurofibromatosis Foundation Meeting, Aspen, CO.

Animal Resources: None

CONCLUSIONS

Work on this research project has been conducted in a timely fashion with very good progress. In vivo and in vitro models were developed to examine the possible differences in angiogenic responses in Nf1 heterozygous mice. To obtain essential proof of concept data, we focus more on the in vivo analysis and have mainly completed our aims. Results indicate that the in vivo neoangiogenic response to hypoxia is heightened in retinas of Nf1+/- mice compared to wild-type mice. This finding was corroborated in a second in vivo model and we found that the in vivo neoangiogenic response to FGF-2 is heightened in the corneas of Nf1+/- mice compared to wild-type mice. Based on these findings, more specific and controlled testing using endothelial cell cultures is clearly warranted. We developed methods to culture brain microvessel endothelial cells from Nf1 and wild-type mice for in vitro angiogenic assays.

We have made excellent progress in aims to establish reliable procedures for xenografting of human NF1 cell lines in the mouse. Thus, far, we established and documented two valid xenograft models of NF1 plexiform neurofibroma and malignant peripheral nerve sheath tumors. using these models tumor growth and vascularity of NF1 tumor xenografts has been quantified by advanced MRI, gadolinium permeability and dynamic contrast enhancement. Corroborative histological measures are in progress.

In summary, the work and aims of this project are mainly proceeding on schedule. Definitive findings were made in the in vivo models of neovascularization and MRI assessment of tumor growth and vascularity. Work on other aims is progressing well.

REFERENCES

None

APPENDICES

None